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STUDIES IN NON-SPECIFIC COMPLEMENT FIXATION *

I. NON-SPECIFIC COMPLEMENT FIXATION BY NORMAL RABBIT SERUM

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The object of this communication is to direct attention to the observation that in complement-fixation tests when normal rabbit serum is used, according to the usual technic, in an active condition or after heating at 55 C. for half an hour for the purpose of inactivation, non-specific complement absorption or fixation may occur, not only in the presence of lipoidal antigens, but with various bacterial antigens as well. This is a matter of considerable importance in view of the fact that rabbits are commonly employed for experimental studies in syphilis, and likewise for the preparation of immune amboceptors for the study and differentiation of bacterial species and proteins in general. This possibility of non-specific complement fixation is to be remembered and reckoned with in complement-fixation work with rabbit serum, especially in experimental syphilis, as otherwise erroneous conclusions may be drawn, if based alone upon complement-fixation tests.

HISTORICAL REVIEW

Schilling and Hoesslin[1] were probably among the first investigators to observe that normal rabbit serum may absorb or fix complement with alcoholic extracts of syphilitic liver; Manteufel and Woithe[2] and Browning and McKenzie[3] have also noted the phenomenon during studies in experimental trypanosomiasis. Dohi[4] examined the sera of 74 normal rabbits, using as antigen an alcoholic extract of syphilitic liver, and found that 39 reacted positively and 35 negatively. He also observed that heated serum was more likely to show this non-specific absorption of complement than unheated serum. Browning and McKenzie, however, state that they have not observed this phenomenon when using serum in a fresh, or active, condition. Blumenthal[5] has likewise observed positive reactions with normal rabbit serum, using an alcoholic extract of syphilitic liver as antigen; Craig and Nichols[6] on the other hand have reported

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[1] Deutsch. med. Wchnschr., 1908, 34, p. 1422.

[2] Centralbl. f. Bakteriol., R., 1909, 43, p. 359.

[3] Jour. Path. and Bacteriol., 1909-1911, 15, p. 182.

[4] Beitr. z. path. u. Therap. der Syph., 1911, p. 514.

[5] Berl. klin. Wchnschr., 1911, 48, p. 1462.

[6] Jour. Exper. Med., 1911, 14, p. 206.

uniformly negative results with an alcoholic extract of syphilitic liver. Similar observations on this power of normal rabbit serum to absorb complement in the presence of lipoidal antigens have been made by Emmanuel[7] and by Epstein and Pribram,[8] the former stating that the administration of salvarsan removes this property temporarily and the latter making similar claims for the mercurials.

Kolmer with Casselman[9] studied this subject, testing the sera of 117 normal and apparently healthy rabbits with antigens comprising alcoholic extracts of syphilitic liver, extracts of acetone insoluble lipoids, alcoholic extract of beef heart re-enforced with cholesterin, and aqueous and alcoholic extracts of a pure culture of Treponema pallidum. Inactivated serum was used throughout. After heating the sera at 55 C. for half an hour, doses varying from 0.05 to 0.4 c.c. being employed, the following results were obtained:

(*a*) In doses of 0.05 c.c. serum, complement fixation did not occur with any of the antigens employed.

(*b*) In doses of 0.1 c.c. serum, complement fixation occurred in 13% of sera with alcoholic extracts of syphilitic liver and in 20% with extracts of beef heart re-enforced with cholesterin.

(*c*) In doses of 0.2 c.c., fixation occurred in 20% of sera with alcoholic extracts of syphilitic liver; in 33% with extracts of acetone insoluble lipoids; and in 40% with cholesterinized alcoholic extracts of heart. With larger doses of serum, as 0.4 c.c., the percentage of complement fixations with the various antigens was even higher.

We have examined the sera of a number of apparently normal rabbits in both the fresh or active, and heated or inactivated state, not only with various lipoidal antigens commonly used in the Wassermann syphilis reaction, but with several indifferent bacterial antigens as well, and we believe that these results are of such importance and significance as to merit a brief report.

METHODS OF STUDY

Sera.—The sera of 75 unused and apparently normal rabbits were used. These animals were kept on a diet composed largely of cabbage, oats, and hay; blood was secured from time to time from an ear vein, and, as a general rule, all sera were tested within 24 to 48 hours, both in an active and inactivated state.

Antigens.—The following three lipoidal extracts were used: alcoholic extracts of human and beef heart re-enforced with cholesterin; an alcoholic extract of syphilitic liver; an extract of acetone insoluble lipoids from human heart. These lipoidal extracts were the same as those used in the regular Wassermann syphilis reactions and were used in the same dosage; namely, twice the antigenic unit as determined by titration with the sera of several luetic persons. These doses were at least 6 to 12 times less than the anticomplementary units as determined by frequent titrations.

For the preparation of bacterial antigens we used the following three micro-organisms: 3 strains of Staphylococcus pyogenes aureus; 1 strain of

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[7] Berl. klin. Wchnschr., 1911, 48, p. 2335.

[8] Ztschr., f. exper. Path. u. Therap., 1909, 7, p. 549.

[9] Jour. Med. Research, 1913, 28, p. 369.

TABLE 1

NUMBER AND PERCENTAGE OF POSITIVE REACTIONS WITH ACTIVE AND INACTIVATED RABBIT SERA AND VARIOUS LIPOIDAL AND BACTERIAL ANTIGENS

Antigens	Active Serum			Inactivated Serum		
	No. Positive	No. Negative	Percentage Positive	No. Positive	No. Negative	Percentage Positive
Cholesterinized alcoholic extract of heart	11	62	15	37	38	49.3
Alcoholic extract of syphilitic liver	6	67	8.2	30	43	41
Acetone insoluble lipoids	4	69	5.4	28	46	38.3
Staphylococcus	15	20	42.8	22	13	62.8
B. coli	11	24	31.4	18	17	61.4
B. typhosus	13	22	37.1	21	14	60

B. typhosus; 6 strains of B. coli communis from human feces. These microorganisms were not selected for any other reason than that they are easily cultivated and represent common species.

These bacterial antigens were simple suspensions of the washed bacteria, shaken for 24 hours in salt solution, filtered through paper, heated to 60 C. for an hour, and preserved with 0.5% phenol. All of them were titrated each time before the tests proper for their anticomplementary units and used in one-quarter amounts.

TABLE 2

DEGREE OF COMPLEMENT FIXATION WITH THE VARIOUS LIPOIDAL AND BACTERIAL ANTIGENS WIT ACTIVE AND INACTIVATED NORMAL RABBIT SERA

Antigens	Active Sera				
	No. Positive	Less than 25% Inhibition	25% Inhibition	50% Inhibition	75% Inhibitio
Cholesterinized alcoholic extract of heart	11	1	2	5	1
Alcoholic extract of syphilitic liver	6	0	2	1	2
Acetone insoluble lipoids	4	0	2	1	0
Staphylococcus	15	1	2	5	5
B. coli	11	3	4	2	0
B. typhosus	13	3	3	3	4

The dosage of either a lipoidal or bacterial antigen bears an important relation to the occurrence and degree of complement absorption, for larger doses of antigen, as one-half the anticomplementary unit, will yield a higher percentage of positive reactions than a dose representing one-quarter or less of this unit.

Technic.—As a general rule, the sera were used in a constant dose of 0.1 to 0.2 c.c.; when inactivated a temperature of 55-56 C. was employed with an exposure of one-half hour. With 0.2 c.c. serum, the percentage of positive reactions and degree of complement absorption were considerably higher than those occurring with 0.1 c.c.

For complement, the pooled sera of 2 or more guinea-pigs were used, diluted 1:20 with normal salt solution, and employed in a constant dose of 1 c.c. The sheep hemolytic system was used throughout, the hemolytic amboceptor being titrated each day with this dose of complement and 1 c.c. of a 2.5% suspension of washed cells, and used in the main tests in a dose equal to 2 hemolytic units. In this manner the hemolytic system is adjusted for each complement serum and erythrocyte suspension. As will be pointed out later, the technic is a matter of importance, as a greater excess of complement or hemolysin yields a lower percentage of positive reactions, whereas with the single unit system the percentage of positive reactions becomes much higher.

Serum complement and antigen with sufficient normal salt solution were incubated for an hour at 37 C.; 2 units of hemolysin and 1 c.c. of the corpuscle suspension were then added. After a second incubation of from 1 to 2 hours, the readings were made. As usual a serum control was used in every instance; likewise antigen, hemolytic, complement, and corpuscle controls as is our custom in conducting complement-fixation tests with human serum. In all tests the sera were used in a constant dose of 0.1 c.c.

SUMMARY OF RESULTS

As shown in the summary of these tests (Table 1) complement absorption under the technic described, with the use of fresh, active, normal rabbit serum and lipoidal extracts, was found to occur in from 5 to 15% of sera; when these same sera were inactivated by heating them in a water bath at 55-56 C. for 30 minutes, complement absorption was observed in 38-49% of sera.

TABLE 2—*Continued*

gree of Complement Fixation with the Various Lipoidal and Bacterial Antigens with Active and Inactivated Normal Rabbit Sera

		Inactivated Sera						
100% hibition	50% or Less Inhibition	No. Positive	Less than 25% Inhibition	25% Inhibition	50% Inhibition	75% Inhibition	100% Inhibition	50% or Less Inhibition
3	72	37	2	5	12	6	13	51.3
1	50	30	0	5	15	3	7	66.6
1	75	28	5	5	10	2	6	71.4
2	53.3	22	0	1	5	5	11	27.3
2	81.8	18	0	9	6	1	2	83.3
0	84.6	21	0	1	7	2	11	38.1

Of equal, or even more, significance is the observation that with various bacterial antigens fresh, active, normal rabbit serum showed some degree of complement absorption in 31-42% of sera; inactivated sera in 51-62%.

As shown in Table 2, the degree of complement absorption with lipoidal extracts was usually moderate or slight, in that 50-75% of sera showed 50%, or less, inhibition of hemolysis. Extracts re-enforced with cholesterin are most likely to react strongly when they react at all.

Bacterial antigens showed even slighter degrees of complement absorption, in that 50-85% gave 50%, or less, inhibition of hemolysis.

CONSTANCY OF RABBIT SERUM IN RELATION TO NON-SPECIFIC COMPLEMENT FIXATION

In order to determine whether the reaction with a rabbit serum was permanent or subject to change under natural conditions, 15 rabbits were examined at intervals of 3 days to a month. Of these, 12 rabbits showed persistently negative or persistently positive results. Three showed varying results as shown in Table 3. As must be expected among the positively reacting animals, very slight differences in the degrees of inhibition of hemolysis were noted at different times, as, for example, a ++ reaction becoming a + or a +++ a week later and subsequently ++ again, or even ++++, but a positively reacting rabbit tends to remain so, as is likewise true with a negatively reacting rabbit.

TABLE 3

VARIATION IN REACTION OF THE SAME RABBIT SERUM AT DIFFERENT TIMES

Serum	Active Serum (0.1 c.c.)				Inactivated Serum (0.1 c.c.)			
	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids	Serum Control	Cholesterinized Alcoholic Extract of Beef Heart	Alcoholic Extract of Syphilitic Liver	Acetone Insoluble Lipoids	Serum Control
19	—	—	—	—	—	—	—	—
19	—	—	—	—	++	++	++	—
19	—	—	—	—	—	—	—	—
19	—	++	—	—	—	++	—	—
6	++	—	—	—	++++	++++	++++	—
6	—	—	—	—	++++	++++	++++	—
23	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—
23	0	0	0	0	++	++++	+	—
23	0	0	0	0	++++	++++	+++	—
35	—	—	—	—	++++	++	++	—
35	—	—	—	—	+++	+++	+++	—
35	—	—	—	—	±	±	—	—
35	0	0	0	0	++++	++++	++++	—
35	0	0	0	0	++++	++++	++++	—
35	—	—	—	—	++	+++	++	—

DISCUSSION

An adequate explanation of this phenomenon of non-specific complement fixation with normal rabbit serum has not been made. As already stated, coccidiosis bears no relation to the condition. It is to be remembered that dosage of serum, kind and dosage of antigen, the degree of temperature and length of exposure in inactivation, and especially the adjustment of the hemolytic system, are factors modifying the occurrence and degree of this non-specific complement absorption. These and other factors that have been subjected to study will be discussed in a separate paper; here it may be stated that while it is well known that the serum alone or an antigen alone may under certain circumstances show non-specific complement absorption, being the so-called "anticomplementary" action of serum or antigen, we are certain that in the technic employed these factors have been adequately controlled and that the results cannot be ascribed to anticomplementary action of serum or to the employment of a too closely adjusted hemolytic system. Indeed, all these examinations were made bi-weekly along with a large number of Wassermann reactions with human sera in which the same lipoidal antigens and same hemolytic systems were employed. The phenomenon is of practical interest and importance, not only from the standpoint of complement-fixation tests in experimental studies in rabbits, but also because a study and explanation of its mechanism may throw more light upon the nature of the syphilis reaction.

We believe that this property of rabbit serum of absorbing or fixing complement in a non-specific manner should be emphasized and better known, for when this animal is used for the purposes of immunization with the object of subsequently conducting complement-fixation tests with the serum, the factor of non-specific complement fixation enters and may greatly modify the interpretation of results. This is of importance not only in experimental studies, but in medico-legal work, as in the differentiation and identification of proteins by complement-fixation tests. It is our custom to test rabbits one or more times before immunization, preferably with the antigens to be subsequently used in the complement-fixation tests, and select those that show a persistently negative reaction.

SUMMARY

Fresh, active sera from normal rabbits in doses of 0.1 c.c. showed non-specific complement fixation with lipoidal extracts in 5-15% of sera.

When these same sera were inactivated by heating them in a water bath at 55-56 C. for 30 minutes, complement fixation occurred in 38-49% of sera.

With both active and inactivated sera the highest percentage of positive reactions was observed when alcoholic extracts of heart muscle re-enforced with cholesterin were used as antigens; alcoholic extracts of syphilitic liver gave the second highest, and an extract of acetone insoluble lipoids gave the lowest, percentage of positive reactions.

With bacterial antigens of staphylococci and colon and typhoid bacilli, fresh, active rabbit sera showed some degree of complement fixation in 31-42% of instances; when these sera were inactivated, positive reactions occurred in 51-62%.

The degree of complement fixation with normal rabbit serum and lipoidal and bacterial antigens is usually moderate or slight, as about 50-85% of reactions show 50%, or less, inhibition of hemolysis.

The reacting state of a rabbit's serum when the animal is on an average diet is usually constant, in that 80% of our animals reacted persistently negatively or persistently positively when examined at intervals of 3 days to a month.

It is to be emphasized that when rabbits are employed for experimental studies with a view to using their sera for complement-fixation tests, their sera should be tested one or more times, before inoculation, preferably with the particular antigen to be used, and only those selected that react negatively.